

Effects of Repeated Administration of Inhaled Anesthetics on Anesthesia, Brain Acetylcholinesterase, Muscarinic Receptors and Dopaminergic Receptors in Mice

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Abstract

There is a lack of information on the effects of repeated administration of volatile anesthetics. Effects of repeated inhalation of enflurane, halothane, isoflurane and sevoflurane, on anesthetic effects and brain neurochemicals were investigated in mice. A simplified but quantitative anesthetic apparatus was employed which estimated the minimal alveolar concentration (MAC, ED₅₀) of enflurane, halothane, isoflurane and sevoflurane to be 1.51, 1.29, 1.10 and 1.65 % (at 1 atm), respectively. Test compounds were administered by inhalation until complete anesthesia was established and then kept for one additional min at 1.73 or 2.16 MAC enflurane (0.4 or 0.5 ml), 1.61 or 2.69 MAC halothane (0.3 or 0.5 ml), 1.38 or 2.30 MAC isoflurane (0.3 or 0.5 ml) or 1.88 or 2.36 MAC sevoflurane (0.4 or 0.5 ml) once per day for 5 consecutive days, recording induction time and recovery time. In addition, rotarod performance (see "Materials and Methods") was tested each day prior to administration of anesthetic. Twenty four hours after the last administration of anesthetic, the brains were removed and acetylcholinesterase (AChE) activity, [³H]quinuclidinyl benzilate (QNB) binding to muscarinic acetylcholine receptor (mAChR) preparation and [³H]haloperidol binding to dopaminergic receptor (D₂-R) preparation in the striatum, cerebral cortex and/or hippocampus were measured. All drugs produced a dose-dependent rapid induction. However, halothane at 1.61 MAC and sevoflurane at 1.88 MAC

produced prolonged induction times after repeated administrations. Enflurane at 1.73 MAC shortened and sevoflurane at 1.88 MAC prolonged the recovery times after repeated treatment. There were no changes in AChE activity in the three brain regions produced by any anesthetic tested. However, enflurane at 2.16 MAC and sevoflurane at 2.36 MAC reduced striatal [³H]QNB binding and isoflurane at 2.30 MAC reduced cerebral [³H]haloperidol binding. In summary, repeated administrations of halothane and sevoflurane may yield some degree of tolerance and repeated use of enflurane and sevoflurane appear to alter receptor kinetics of mAChRs and D₂-R in specific brain areas.

Keywords

Acetylcholinesterase; Dopaminergic Receptors; Enflurane; Halothane; Isoflurane; Muscarinic Receptors; Sevoflurane

Introduction

Inhaled anesthetics such as enflurane, halothane, isoflurane and sevoflurane are used in human and animal surgery to provide anesthesia, analgesia, and immobility or as preanesthetics for other agents. It was formerly thought that general anesthetics interact with the lipid bilayer of neuronal membranes, thereby suppressing the operation of membrane ion channels (Kaufman 1977). However, volatile anesthetics appear

to alter synaptic transmission through presynaptic and postsynaptic mechanisms in the central nervous system (CNS) (Griffiths et al. 1993; Evers et al. 2006; Richards 1996). Although the exact mechanism of anesthesia is still unclear, it is possible that volatile anesthetics modulate homeostasis of neurotransmitters such as dopamine and acetylcholine (ACh) including release, receptor function and enzyme activity (Adachi et al. 2001; Shichino et al. 1998; Lintern et al. 2000).

ACh and dopamine are representative neurotransmitters in the CNS and the activity of cholinergic and dopaminergic neurons have been postulated to play a key role in regulating states of consciousness and cognition (Celesia and Jasper 1966; Deutch and Roth 2004). In fact, some studies have suggested that volatile anesthetics modulate ACh and dopamine release (Adachi et al. 2005; Miyano et al. 1993; Naruo et al. 2005; Shichino et al. 1998; Wang et al. 2012; Westphalen et al. 2013) as well as the affect on brain acetylcholinesterase (AChE) activity and acetylcholine receptors (Lintern et al. 2000; Naruo et al. 2005).

Although it is well known that repeated intravenous administrations of general anesthetics such as the barbiturates (e.g., pentobarbital and thiopental) produce tolerance, perhaps by inducing cytochrome P450 (O'Brien 1996), the effects of repeated inhalation of other volatile anesthetics on anesthesia and neurochemical activities have been inadequately studied. Impairment of neurobehavioral performance is also apprehensive for workers exposed often to inhaled anesthetics in operating-theaters (Scapellato et al. 2008; Vouriot et al. 2005). Therefore, it is important to understand more about the repeated effects of inhaled anesthetics in relation to efficacy, tolerance and neurotransmission. Here we report the effects of repeated administrations of enflurane, isoflurane, halothane and sevoflurane in mice on anesthetic efficacy as measured by induction time, recovery time, and brain neurochemical parameters such as AChE activity, muscarinic ACh receptors and dopaminergic receptors.

Materials and Methods

Animals

Female ICR mice (6 weeks old) purchased from Japan SLC (Hamamatsu, Japan), were allowed having free access to food and water in a room kept at $23 \pm 2^\circ\text{C}$

with a 12-h light (8:00-20:00): 12-h dark photoperiod and used 1 to 3 weeks later. Animal handling and procedures were conducted in strict concordance with the Animal Welfare and the Guide for the Care and Use of Laboratory Animals and approved by the Animal Experiment Committee in Iwate University, Japan.

Chemicals

Enflurane, halothane, isoflurane, and sevoflurane were purchased from Abbott Japan (Tokyo), Takeda Pharmaceutical Co. Ltd. (Osaka, Japan), Dainippon Sumitomo Pharma Co. Ltd. (Osaka, Japan) and Maruishi Pharmaceutical Co. Ltd. (Osaka, Japan), respectively. [^3H]quinuclidinyl benzilate (QNB) and [^3H]haloperidol were obtained from New England Nuclear (Boston, MA). Acetylthiocholine iodide was from Wako Pure Chemical Industries (Osaka, Japan) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) from Kanto Chemical Co., Inc. (Tokyo, Japan).

Anesthesia

A simple and precise apparatus was designed and made to rapidly produce a homogeneous inhalation environment. A framed flat dish (19.6 cm²) was attached to the inner wall of a transparent Tupperware container (15.6 x 20.5 x 14.1 cm) which was also fitted with a lid containing a fan and a hole through which a pipette could be inserted (Fig. 1). The fan is connected to a switch and 2 AA-sized batteries put in parallel. A mouse could then be placed in the bottom of the container which could be sealed completely by the modified lid (net volume is about 4,300 ml). An anesthetic was then added to the dish with a micropipette followed by plugging the hole with a rubber plug and immediately turning on the fan and simultaneously starting a stopwatch. The depth of anesthetic layered on the dish was calculated to be 0.15 mm and 0.25 mm when 0.3 ml and 0.5 ml of the anesthetic is added, respectively, and the anesthetic was instantly vaporized (<1 s) and distributed homogeneously by the continuous mixing of the atmosphere by the fan. The mouse was removed from the apparatus after 60 s without movement. The induction time was the time until the mouse became immobile (even if the apparatus was shaken) and the recovery time was the time after the animal was removed from the apparatus until it showed a positive righting reflex. For the neurochemical examinations, control mice were treated in the apparatus in the same manner as described above but for 3 min without inserting any anesthetic. A mouse was placed in the

apparatus and treated with or without (control) an anesthetic once daily for 5 days.

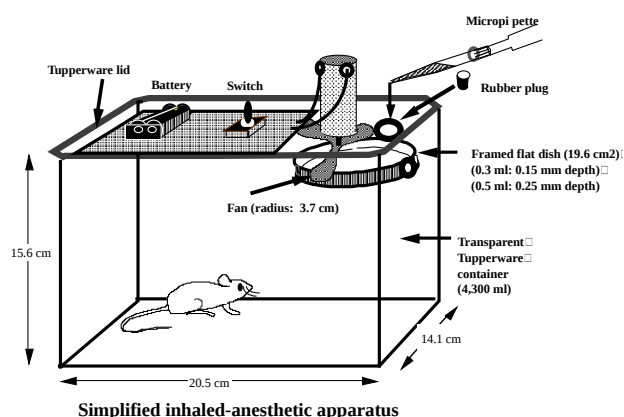


FIG. 1 ANESTHETIC APPARATUS

Rotarod performance

The rotarod performance test is a performance test based on a rotating rod with forced motor activity being applied, usually by a rodent. The mice were placed on a Rotarod (Model KN-75, Natume, Tokyo, Japan, 30 mm in diameter) rotating 5.5 rpm and trained until they were able to stay for longer than 180 s for 2 days before the beginning of experiment. The testing was conducted before anesthesia every day.

Acetylcholinesterase activity

The mice were euthanized with dimethylether 24 h after the last treatment. Three brain regions, striatum, hippocampus and cerebral cortex (cortex) were quickly dissected on ice by the method of Glowinsky and Iversen (1966) and homogenized on ice in 20 to 40 vol (w/v) of ice cold 0.1 M phosphate buffer (pH 8.0) using a Polytron homogenizer (Biotron, Switzerland). The homogenates were further diluted with the same buffer to get 200-fold suspension for the hippocampus and cortex and 400-fold suspension for the striatum. The activity of AChE activity in the homogenate was assayed by the method of Ellman et al. (1961) with 0.48 mM acetylthiocholine iodide as substrate for 2 min at 25°C using UV-240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at 412 nm.

Muscarinic acetylcholine receptors and dopaminergic receptors

Different mice were used for the assays of mAChR and dopamine D₂ receptors (D₂-R). The binding of [³H]quinuclidinyl benzilate (QNB) and [³H]haloperidol to receptor preparations of striatum and cortex was determined to assess mAChR and D₂-R, respectively.

As above, the animals were euthanized 24 h after the last anesthetic treatment, the striatum and cortex were homogenized in 39 vol (v/w) and 19 vol (v/w) of ice-cold 0.32 M sucrose solution, respectively, using a Polytron homogenizer and centrifuged at 1,000 × g for 10 min at 2°C. The supernatants were used as mAChR or D₂-R preparations. The binding of [³H]QNB or [³H]haloperidol to the preparation was assayed after a 40 min incubation of 0.03 ml of the mAChR preparation or D₂-R preparation with 0.237 nM [³H]QNB or 0.833 nM [³H]haloperidol, respectively, in 1.0 ml of Tris-HCl buffer (pH 7.4) at 25°C according to the filtration procedures by Yamamura and Snyder (1974). Total binding and nonspecific binding were determined in the presence or absence of atropine sulfate at 10⁻⁶ M for the bindings of [³H]QNB and in the presence or absence of haloperidol hydrochloride at 10⁻⁶ M for the bindings of [³H]haloperidol. The difference in [³H]QNB bound or [³H]haloperidol bound with and without atropine or haloperidol represents the specific binding (binding ability).

Protein assay

Concentration of protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Statistical analysis

Data are expressed as mean ± SEM (n=5). Differences between groups (p<0.05) were calculated by one-way analysis of variance (ANOVA) followed by Dunnett's test.

Results

Minimum alveolar concentration

In our simplified anesthetic apparatus the values of minimum alveolar concentration (MAC, %, ED₅₀) of enflurane, halothane, isoflurane and sevoflurane were estimated to be approximately 1.51, 1.29, 1.10 and 1.65 % (at 1 atm), respectively, by up-and-down dosing (Lichtman 1998) (Table 1).

TABLE 1 THE VALUES OF MAC OF VOLATILE ANESTHETICS

Anesthetic	MAC (%)	
	Present	Reference*
Enflurane	1.51	1.09-1.95
Halothane	1.29	1.19-1.39
Isoflurane	1.10	1.02-1.77
Sevoflurane	1.65	1.95-2.29

Six to 8 mice were used for each anesthetic

*Sonner et al. 1999; Sun et al. 2006; Tanaka et al. 1993

In the present study 0.4 or 0.5 ml of enflurane and

sevoflurane, and 0.3 or 0.5 ml of halothane and isoflurane were used. These doses corresponded to 1.73 or 2.16 MAC enflurane, 1.89 or 2.36 MAC sevoflurane, 1.61 or 2.69 MAC halothane and 1.38 or 2.30 MAC influrane, respectively.

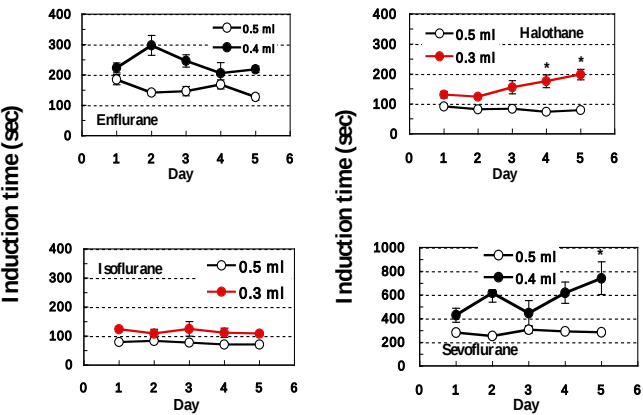


FIG. 2 EFFECTS OF VOLATILE ANESTHETICS ON INDUCTION TIME TO ANESTHESIA

The time in s until a mouse became completely immovable with only breathing was measured. Data represent means ± S.E. (n=5). Significantly different from Day 1 value (*p<0.05).

Effect of repeated administration on anesthesia

Figure 2 shows the effect of repeated administrations of enflurane, halothane, isoflurane and sevoflurane on induction times. A dose-dependent reduction in induction time was produced by all anesthetics for all 5 tested days. However, it is interesting to note that induction times increased following the administration of the lower doses of halothane and sevoflurane, as compared to the first day, but no changes in induction times were observed following both doses of enflurane and isoflurane, and higher doses of halothane and sevoflurane over the 5 days of testing.

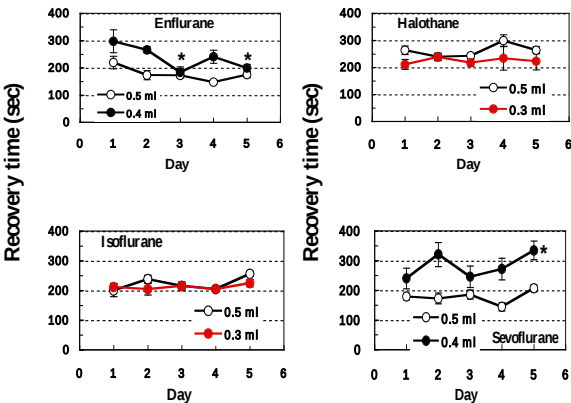


FIG. 3 EFFECTS OF VOLATILE ANESTHETICS ON RECOVERY TIME FROM ANESTHESIA

The time in s until a mouse walked around with complete righting reflex was measured. Data represent means ± S.E. (n=5). Significantly different from Day 1 value (*p<0.05).

As shown in Fig. 3, in contrast to the results obtained with induction times, there was no dose-dependent effect observed on recovery times with the three anesthetic drugs except with sevoflurane wherein recovery from the lower dose took rather longer than that from the higher dose. The recovery times were shorter following the lower dose of enflurane at Days 3 and 5, and prolonged following the lower dose of sevoflurane at Day 5 when compared to Day 1. No significant changes in the recovery time have been observed across the 5 days of inhalation testing at either dose of halothane and isoflurane nor with the higher dose of enflurane and sevoflurane.

Effect of repeated administration on rotarod performance

As described above, rotarod performance was measured before inhalation each day of the 5 days of testing. The time until the mouse fell from the rod or a maximum time of 180 s was recorded. Every mouse remained on the rotarod for the maximum of 180 s for all 5 days (data not shown).

Effect of repeated administration on acetylcholinesterase activity

The activity of AChE in the hippocampus, striatum and cortex was determined 24 h after the last administration of enflurane, halothane, isoflurane and sevoflurane (Table 2). Insignificant change in AChE activity was found in any of the three brain regions at two doses of each anesthetic tested when the respective activity was compared with that of control mice (Table 2 shows the data at 0.5 ml of each anesthetic.).

TABLE 2 EFFECT OF REPEATED TREATMENT WITH VOLATILE ANESTHETICS ON ACETYLCHOLINESTERASE ACTIVITY

Treatment	AChE activity (μmol acetylthiocholine hydrolyzed/g tissue/min)		
	Hippocampus	Striatum	Cortex
Control	13.57 ± 1.07	41.44 ± 7.76	17.01 ± 1.00
Enflurane	10.28 ± 1.92	44.45 ± 4.26	14.65 ± 2.24
Halothane	11.59 ± 2.71	37.82 ± 8.56	12.85 ± 2.74
Isoflurane	11.07 ± 2.06	40.32 ± 5.50	13.81 ± 2.11
Sevoflurane	11.15 ± 2.91	39.26 ± 4.08	16.86 ± 4.08

The activity of AChE in three brain regions was determined 24 h after the last inhalation of each anesthetic for 5 days at a dose of 0.5 ml. Data represent means ± S.E. (n=5)

Effect of repeated administration on dopaminergic receptors

The binding of [3 H]haloperidol to the receptor preparations from the striatum and cortex was assayed 24 h after the last administration of enflurane, halothane, isoflurane and sevoflurane. As shown in Fig. 4, a significant decrease in [3 H]haloperidol binding was produced by the lower dose of sevoflurane in the striatum and by the lower dose of halothane and the higher dose of isoflurane, caused in the cortex. It is also interesting to note that halothane and sevoflurane at the higher doses in the striatum, halothane and sevoflurane at the higher doses in the cortex, and isoflurane at the lower dose in the cortex all showed some decrease in [3 H]haloperidol binding but it was not statistically significant.

Effect of repeated administration on muscarinic ACh receptors

The binding of [3 H]QNB to the receptor preparations in the striatum and cortex was determined 24 h after the last administration of enflurane, halothane, isoflurane and sevoflurane.

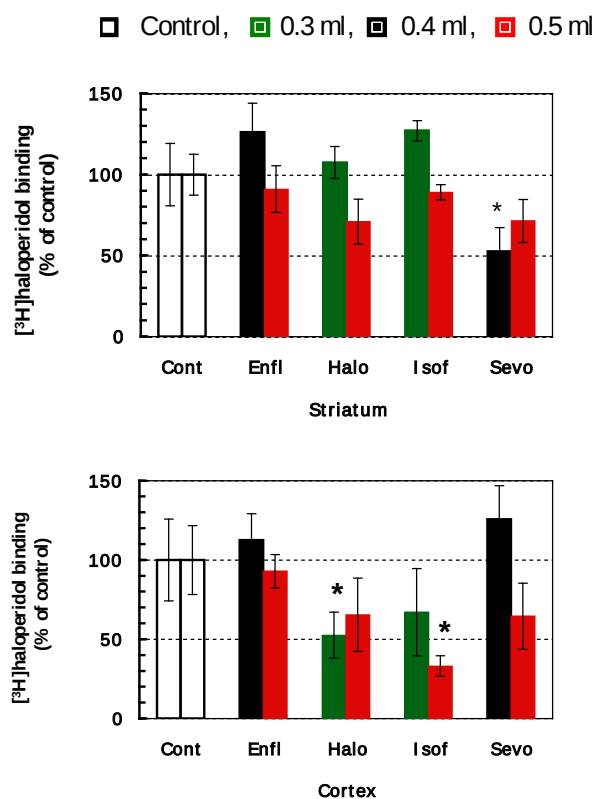


FIG. 4 EFFECTS OF VOLATILE ANESTHETICS ON THE BINDING ABILITY OF [3 H]HALOPERIDOL TO D₂-R PREPARATION IN THE BRAIN REGIONS

The binding ability of [3 H]haloperidol in the striatum

and cortex was determined 24 h after the last inhalation of enflurane (Enf), halothane (Hal), isoflurane (Iso) or sevoflurane (Sev) for 5 days at two doses. Data represent means $\pm$ S.E. (n=5). An asterisk indicates that is significantly different from control values (*p<0.05).

As shown in Fig. 5, the higher doses of enflurane and sevoflurane showed a significant decrease in the binding ability of [3 H]QNB in the striatum. There were no changes in [3 H]QNB in cortex after treatment with any anesthetic tested.

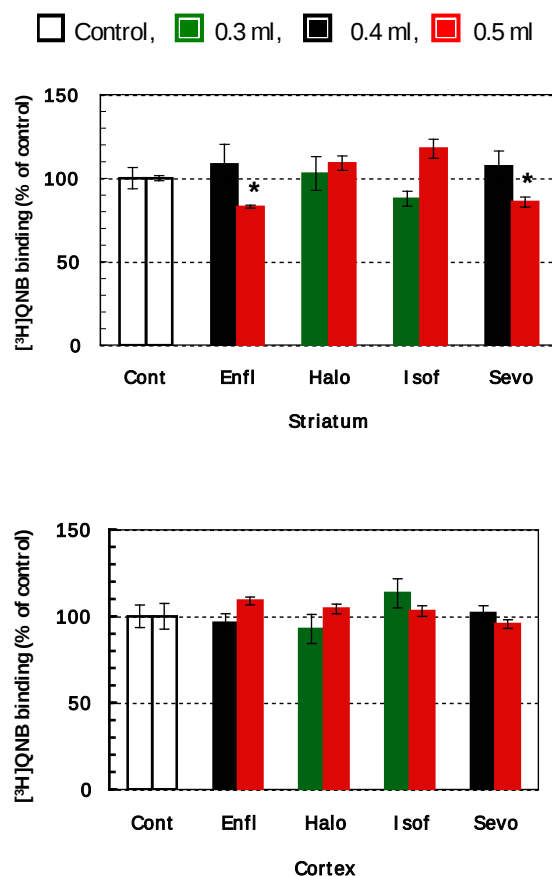


FIG. 5 EFFECTS OF VOLATILE ANESTHETICS ON THE BINDING ABILITY OF [3 H]QNB TO MACHR PREPARATION IN THE BRAIN REGIONS

The binding ability of [3 H]QNB in the striatum and cortex was determined 24 h after the last inhalation of enflurane, halothane, isoflurane or sevoflurane for 5 days at two doses. For further information, see the legends of Fig. 5

Discussion

The MAC values determined for enflurane, halothane, isoflurane, but not sevoflurane, in the present experiment were in the range of the respective values in mice reported in the references (Sonner et al. 1999;

Sun et al. 2006; Tanakaka et al. 1993). The MAC value for sevoflurane in the present experiment was a little lower than that ever reported before (Sonner et al. 1999; Sun et al. 2006; Tanakaka et al. 1993). This may be due to strain differences in anesthetic effects which have been reported in mice (Sonner et al. 1999; Tanaka et al. 1993). There have been no reported MAC values for volatile anesthetics in the ICR strain of mice. It was also interesting that sevoflurane required a comparatively long induction time (e.g., Fig. 2, the induction time of anesthetics at 0.5 ml was about 3.8, 2.2, 2.0 and 7.2 min for enflurane, halothane, isoflurane and sevoflurane, respectively).

The simple apparatus developed and used in these experiments vaporized the anesthetics rapidly and efficiently at comparatively low MAC values. Our MAC values were close to the values reported in other references (Sonner et al. 1999; Sun et al. 2006; Tanaka et al. 1993) and it was demonstrated that our anesthetic apparatus can anesthetize mice reliably and quantitatively. Without the fan, however, the induction time to anesthesia by 0.5 ml of enflurane, halothane, isoflurane and sevoflurane was significantly prolonged by 2.9, 2.0, 2.8 and 2.1 times, respectively, in comparison with to times obtained with the fan. The reliability of the apparatus also depended on the fan. Without the fan, the standard deviation of mean of the induction times was 6 to 10 times larger than times observed with the fan. The fan efficiently vaporized up to 0.5 ml (the maximal volume in the present experiments) of anesthetic within 1 s and diffused the vapor homogeneously and simultaneously throughout the atmosphere within the apparatus. Since the apparatus is transparent and light enough to shake easily, it was convenient to monitor the anesthetic level of the mouse not only to the loss of the righting reflex but also to complete immobilization. It is suggested that our anesthetic apparatus is convenient and appropriate for pharmacological or mechanical investigations of nonflammable volatile anesthetics.

Tolerance is characterized with the need for increasing amounts of a drug to obtain the same effect or a reduction of the drug-induced response following repeated administrations of the test agent. Since repeated administration of halothane and sevoflurane at the lower doses prolonged the induction time to full anesthesia and repeated administrations of enflurane at the lower dose shortened the recovery time, it is suggested that tolerance to their effects may have been induced. It is unclear why these anesthetics at lower

doses, but not higher doses, may have induced tolerance.

Anesthetic-induced unconsciousness is typically assessed using the loss of righting reflex assay (Sun et al. 2006). However, the induction of anesthesia was assessed by the time required to produce complete immobility. The induction time to anesthesia following the lower doses was longer than that following the higher doses. Therefore, total exposure to the anesthetics may have been greater following lower doses compared to that at higher doses. This may also explain that the recovery took rather longer following the lower dose of sevoflurane than the higher dose, and the recovery times were shorter following the lower dose of enflurane at Days 3 and 5, when compared to Day 1 (Fig. 3). Relative to the wealth of reports concerning tolerance to cerebral ischemia and neuroprotection induced by inhaled anesthetics, few studies have been conducted on auto-tolerance following repeated exposures to the anesthetics (Chalon et al. 1983; Smith et al. 1979). In one of the few available reports, Chalon et al. (1983) reported that repeated administration of halothane, isoflurane and sevoflurane produced tolerance in mice.

Rotarod performance is used to access motor coordination, including CNS functions, in rodents. Since none of the anesthetics used at either of the two doses tested did not cause a change in rotarod performance over the 5 days or repeated exposure, it is suggested that repeated anesthesia with volatile anesthetics do not affect motor coordination in mice.

The present study showed that repeated inhalation of enflurane, halothane, isoflurane and sevoflurane at either dose tested did not affect the activity of AChE in the hippocampus, striatum and cortex. Lintern et al. (2000) reported that a single administration of halothane (3% in O₂) for 3 min to guinea-pigs increased the activity of the G4 form of AChE in the medulla-pons and decreased that of G1 in the hippocampus and midbrain 6 days after administration. This conflicting effect suggests that the effect of halothane on AChE activity may be secondary to other actions. As with our results, however, Lintern et al. (2000) found that the total activities of AChE in the hippocampus, striatum and cortex were unchanged. Therefore, brain AChE activity may not be significantly involved in the effects of volatile anesthetics.

In the present study, repeated treatment with volatile

anesthetics reduced [^3H]haloperidol binding in the striatum and cortex. It is generally known that inhibition of receptors is produced by direct interference with the drug or down-regulation. Down-regulation of receptors is believed to occur after prolonged exposure of receptors to the agonist. It has been reported that volatile anesthetics such as isoflurane and halothane inhibited a high-affinity state of D2 receptors in vitro and in vivo (Seeman and Kapur 2003). On the other hand, volatile anesthetics such as isoflurane did not affect D2 receptors (Nader et al. 1999; Tanifuji et al. 2006). It has also been reported that volatile anesthetics increase DA release in the brain in vitro and in vivo (Silva et al. 2007; Adachi et al. 2005). It is likely from these findings that repeated administration of volatile anesthetics may affect D2 receptors mainly by down-regulation.

It was also found that repeated inhalation of enflurane and sevoflurane at the higher doses decreased [^3H]QNB binding in the striatum. Isoflurane and halothane, and enflurane and sevoflurane at lower doses did not affect [^3H]QNB binding in the striatum and cortex. It has also been reported that volatile anesthetics such as enflurane, isoflurane, halothane and sevoflurane may affect mAChRs in vitro in a different manner (Lin et al. 1993; Nietgen et al. 1998). In fact, Anthony et al. (1989) have proposed that interference with muscarinic receptor interactions was a common property of liquid volatile anesthetics and may represent a general mechanism for the disruption of signal transmission between cells during anesthesia. While halothane and sevoflurane inhibited both m1 and m3 mAChRs, isoflurane had no effect on m1 mAChRs but it inhibited m3 mAChRs (Anthony et al. 1989). The chemical structure of these various anesthetics are similar but Anthony et al. (1989) propose that these minor changes in structure can significantly alter their pharmacological effects. It is well known that there are five identified subtypes of mAChRs (m1-m5) in rodent brain and the expression of m1, m2 and m3 mAChRs is abundant (up to 40-50% of the total mAChRs), markedly lower (12-20%) and much lower (only 5-10%), respectively (Abrams et al. 2006; Levey et al. 1991). This may explain that isoflurane did not induce a significant change in the binding ability of [^3H]QNB, which is a nonspecific muscarinic ligand.

Conclusion

The present investigation has provided the first report

on the effects of repeated administration of 4 volatile anesthetics, enflurane, halothane, isoflurane and sevoflurane, on anesthesia and brain neurochemical events with a simplified but quantitative anesthetic apparatus. The MAC values determined for the anesthetics other than sevoflurane were in the range of the respective values in mice reported in the references. A kind of tolerance, which was estimated by recording induction time and recovery time, to halothane, sevoflurane and enflurane was shown following repeated inhalation in mice. Since none of the anesthetics used at the doses tested did not cause a change in rotarod, it is suggested that repeated anesthesia with volatile anesthetics do not affect motor coordination in mice. The repeated inhalation of all anesthetics at dose tested did not affect the activity of AChE in the hippocampus, striatum and cortex. The binding of [^3H] QNB and [^3H]haloperidol to receptor preparations of striatum and cortex was determined to assess mAChR and D2-R, respectively. Repeated treatment with some volatile anesthetics reduced [^3H]haloperidol binding in the striatum and cortex. Repeated inhalation of enflurane and sevoflurane at the higher doses decreased [^3H]QNB binding in the striatum. It seems likely that brain cholinergic and dopaminergic receptors are affected by the chronic treatment with some volatile anesthetics. Further study is expected to clarify the relationship between the changes of neurotransmission such as dopaminergic and muscarinic nervous system and the changes of anesthetic action after the repeated administration of volatile agents.

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